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Comet Assay results of pilots exposed to pesticides

Resultados del Ensayo Cometa en pilotos expuestos a plaguicidas

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ABSTRACT

Pesticides constitute a heterogeneous category of chemicals designed for the control of pests affecting cultivated plants. Frequently, they are classified according to their chemical structure, organic and non-organic pesticides. Biomonitoring studies using somatic cells have been conducted to evaluate the possible genotoxic risk of occupationally exposed workers to pesticides. The aim of this study was to assess the genotoxic effects of pesticides in pilots occupationally exposed to these chemicals during aerial application in agricultural fields. The study groups comprised 30 pilots who applied aerial pesticides and 30 controls. The alkaline Comet Assay was performed on freshly collected peripheral whole blood lymphocytes. The nonparametric Mann-Whitney test was applied to compare the equality of two population medians. Additionally, a comparison of two groups according to age and years of work as quantitative variables and a one-way analysis of variance (Anova) with Tukey's post hoc test were applied. To corroborate differences between groups, a regression analysis was performed to calculate the degree of correlation, expressing their magnitude by R^2. Statistical significances were set at a p value of <0.05. The median of comet frequency, tail length (159.6 $\pm$ 16.8) and tail moment (16.75 $\pm$ 3.13) reveals statistically significant differences ($p < 0.001$) between exposed pilots and controls. The pilot group divided according to age reveals Deoxyribonucleic acid (DNA) damage which increases significantly when age of participant increases. Neither smoking nor alcohol consumption could be statistically linked to DNA damage.

RESUMEN

Los plaguicidas constituyen un grupo heterogéneo de químicos destinados para control de plagas que afectan plantas cultivadas. Frecuentemente se clasifican en función de su estructura química, en plaguicidas no orgánicos y orgánicos. Estudios de biomonitoreo utilizando células somáticas han conducido a la evaluación del posible riesgo genotóxico en trabajadores expuestos a plaguicidas. El objetivo de este estudio fue evaluar los efectos genotóxicos de plaguicidas en pilotos ocupacionalmente expuestos a estos durante su aplicación aérea en campos agrícolas. Se analizaron linfocitos de sangre periférica recién recolectada de 30 pilotos que aplican plaguicidas vía aérea y 30 controles, mediante electroforesis unicelular alcalina (ensayo cometa). La prueba U de Mann Whitney se aplicó para determinar el grado de asociación de los resultados del ensayo Cometa entre los pilotos expuestos y el grupo control. Un análisis de varianza (Anova) de una sola vía y una prueba de Tukey's post hoc fueron utilizadas para comparar los dos grupos de acuerdo a edad y años de exposición ocupacional. La correlación entre los grupos se analizó a través de regresión logística expresado por R^2. Un valor de probabilidad ≤ 0.05 se consideró estadísticamente significativo. La frecuencia de la mediana en la longitud de cola fue (EE:159.6 $\pm$ 16.8) y el momento de la cola (EE:16.75 $\pm$ 3.13) revelan diferencias estadísticamente significativas ($p < 0.001$) entre pilotos y controles. El grupo de pilotos revela frecuencias elevadas de daño al Ácido Desoxirribonucleico (ADN), el cual se incrementa significativamente al aumentar la edad de los participantes, pero no se correlaciona con el consumo de alcohol y tabaco.

INTRODUCTION

Global concern about the use of agricultural chemicals has grown significantly during the past few years. In particular, the relationship between both health and environmental issues and pesticide dosage is a persistent problem in rural and urban areas. One of the main causes of this problem could be the transport of pollutants via different pathways: air, water, foods and feeds (Gil & Sinfort, 2005).

Pesticides constitute a heterogeneous category of chemicals designed for the control of pests, and their application is the most used tool for the protection of plants from plagues and have significantly contributed to the enhanced agricultural productivity (Bolognesi, 2003). In many countries with extensive agricultural production, an increasing trend in crop cultivation is observed, which involves widespread use of pesticides during vegetation periods. Unfortunately, pesticide residues can be found in air, soil, water, plants and harvested products, as well as on application equipment, on the clothing of agricultural workers, and in human and animal tissues. Once dispersed, pesticides can enter the human body by swallowing, by breathing, or directly through the skin. Toxic effects in an exposed population depend on individual health status and sensitivity to a particular pesticide (Bolognesi, 2003; Gaikwad, Karunamoorthy, Kondhalkar, Ambikapathy & Beerappa, 2015). Therefore, while pesticides have brought revolutionary changes to agriculture, they also represent a major potential health occupational hazard for agricultural workers (Bhalli, Khan, Haq, Khalid & Nasim, 2006; Bolognesi, 2003).

Disregarded use of agricultural chemicals runs the risk of induced mutations and the possible development of some types of tumors (Yaduvanshi, Srivastava, Marotta, Jain & Yadav, 2012). Other known human health hazards include mild headaches, flu, skin rashes, blurred vision and other neurological disorders; while rare, severe human health hazards may include paralysis, blindness and even death (Abhilash & Singh, 2009).

Aerial application is the most frequent application method associated with drift events, and drift hazards from aerial applications have been well documented (Weppner *et al.*, 2006). Pesticide drift, the off-target movement of pesticides, is recognized as a major cause of pesticide exposure affecting people involved in agricultural practices and inhabitants living in communities situated close to protected fields (Lee *et al.*, 2011). The occurrence and extent of pesticide drift are affected by many factors such as the nature of the pesticide (e.g., fumigants are highly volatile, which increases their propensity for off-site movement), the equipment and application techniques (e.g., size and height of the spray nozzles), the amount of pesticide applied, and the weather (e.g., wind speed, temperature inversion), and the operator care (Lee *et al.*, 2011). Pesticide applicators are required to use necessary preventive measures and to comply with label requirements to minimize pesticide drift and risk of pesticide exposure; however, they do not often comply with all this.

Biomonitoring studies using somatic cells have been extensively conducted to evaluate the possible genotoxic risk of a defined exposure, and some indicators, such as chromosomal aberrations and abnormalities, have been shown to be a relevant biomarker for predicting cancer risk (Hagmar *et al.*, 1998; Martínez-Valenzuela *et al.*, 2015). A certain number of field studies have been carried out, revealing an association between the occupational exposure to pesticides and the presence of chromosomal aberrations and AND damage (Bolognesi, Parrini, Bonassi, Ianello & Salanitto, 1993; Cuenca & Ramírez, 2004; Garaj-Vrhorac & Želježić, 1999; Gómez-Arroyo *et al.*, 2013; Rupa, Rita, Reddy & Reddi, 1998).

Chemical, physical and biological agents can interact with the genetic material, resulting in mutations, which are associated to genomic instability and cancer. Accordingly, genotoxicity testing represents an essential part of chemical validation. Such tests should include *in vitro* and *in vivo* assays to detect the pesticide potential to induce genetic mutations and/or chromosomal aberrations (Araldi *et al.*, 2015). Among the available genotoxicity tests, the Comet Assay is recognized due to its robustness, sensitivity and statistical power to evaluate Deoxyribonucleic acid (DNA) breaks, which can be considered as hallmarks of mutagenicity (Araldi *et al.*, 2015). The alkaline Comet Assay can evaluate cellular single and double-stranded DNA breaks induced by pesticide exposition and determine the cytotoxic effect of pesticides by assaying for apoptosis-associated fragmentation of nuclear DNA.

In this study, we have used the Comet Assay to assess the potential health risks associated with daily exposure to pesticides used most frequently in harvest fields of Sinaloa, Mexico, which are listed in table 1. Therefore, the purpose of the study was to assess the genotoxic effects of pesticides in workers occupationally exposed to these chemicals during their manipulation and aerial applications in agricultural fields of Sinaloa, Mexico.

Table 1 Pesticides most frequently used in Sinaloa, Mexico

Pesticide	
Organochlorine	Endosulfan, Pentachlorphenol, Quintozen
Organophosphorus	Chlorpiriphos, Dimethoate, Malathion, Monocrotophos, Methyl parathion
Carbamates	Aldicarb, Carbofuran, Methomil, Oxamil, Benomil, Mancozeb, Thiram, Metam
Piretroids	Betacifluthrin, Bifentrin, Lambda-Cihalothrin, Cipermethrin, Deltamethrin, Permethrin, Zeta-Cipermethrin
Neonicotinoids	Acetamiprid, Clothianidin, Imidacloprid, Tiametoxam
Triazins	Ciromazin, Atrazin
Triazols	Diphenoconazol, Epoxiconazol , Propiconazol, Tebuconazol
Others	Abamectrin, Novaluron, Sulfoxaflor, 2,4-D, Dicamba, Gliphosate, Nicosulfurón, Paraquat, Azoxystrobin, Boscalid, Captan, Carbendazim, Carboxin, Clorothalonil, Cymoxanil, Dimethomorf, Fluazinam, Fluoxastrobin, Alumino Fosetil, Mandipropamid, Metalaxil, Cuprum oxide, Procloraz, Pyraclostrobin, Tiabendazol, Methyl Tiophanate, Trifloxystrobin, Methyl bromide

Source: Author's own elaboration.

MATERIALS AND METHODS

Participants

The study groups were made from the population working as pesticide applicators in Sinaloa, Mexico. The groups consisted of 30 pilots of small airplanes used for pesticide aerial dispersions and 30 unexposed control individuals who are engaged in other tasks and reported no history of occupational exposure to pesticides. Both groups were matched according to age, years of work as pesticide applicators, and lifestyle habits such as smoking and alcohol consumption in order to calculate whether the obtained results of abnormal nuclear Comet Assays could be influenced by the pesticides applicators' lifestyle. This study was approved by the Ethics Committee of Universidad de Occidente, and all participants involved gave their consent prior to intervention.

Sample collection

Peripheral blood lymphocytes were separated from freshly collected whole blood by centrifugation and washed twice in phosphate-buffered saline.

Cell Viability Test

Cell viability was estimated before using the trypan blue exclusion method (Altman, Randers & Rao, 1993). Trypan blue penetrates the damaged membrane of dead cells and stains the nucleus. A mix of 10 µL of cell pellet and 10 µL of trypan blue was incubated for 3 min. Then the number of dead cells out of 100 consecutive cells was counted in duplicate.

Alkaline Comet Assay

The alkaline Comet Assay was performed according to procedures previously described by Singh, McCoy, Tice & Schneider (1988) and Tice *et al.* (2000). Briefly, lymphocytes (50 µL) were mixed with 100 µL of low-melting-point agarose (0.5%) at 37 °C, placed on fully frosted slides (Fisher) coated with a thin layer of normal-melting-point agarose (1%) and covered with a coverslip. Two slides were made for each individual. The slides were kept at 4 °C for 5 min to allow the agarose to solidify. The coverslip was then carefully removed, and the slides were immersed in a Coplin staining jar containing a freshly prepared cold lysis solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, 1% Triton X-100, and 10% DMSO, pH = 10) at 4 °C for 1 h. The slides were placed in a horizontal electrophoresis chamber (Owl A5, Lab System Inc.) containing freshly prepared cold electrophoresis alkaline buffer (300 mM NaOH, 1 mM EDTA, pH = 13) for 20 min to unwind the DNA. Electrophoresis was carried out at 25 V and 300 mA for 20 min in darkness to prevent additional DNA damage. The slides were then washed three times with freshly prepared neutralization buffer (0.4 M Tris, pH 7.5) for 5 min, fixed with cold absolute methanol for 5 min and air-dried at room temperature. Next, 50 µL of ethidium bromide (20 mg/mL) was added to each slide to stain the DNA. The slides were labeled with a code that was unfamiliar to the viewer and examined with an Axiostar Plus Carl Zeiss fluorescent microscope equipped with an excitation filter (515 nm - 560 nm) and a barrier filter (590 nm).

Statistics

Statistical analyses were conducted using the Minitab statistical software version 12 (Minitab Inc. State College, PA, USA), where continuous variable values of DNA damage were expressed by descriptive statistics as frequencies of their presence, such as arithmetic mean $\pm$ standard errors of mean (SEM) and median. In order to determine the degrees of associations between results of Comet Assay in pilots exposed to pesticides and the control group, the nonparametric Mann-Whitney test was applied, which compares the equality of two population medians. Additionally, comparison of two groups according to age and

years of work as quantitative variables, one-way analysis of variance (ANOVA) with Tukey's post hoc test were applied. To corroborate differences between groups, a regression analysis was performed to calculate the degree of correlation expressing their magnitude by R^2. Statistical significances were set at a p value of <0.05 and marked with an asterisk. Moreover, to visualize the magnitude of differences between the genotoxic results of exposed pilots and controls, the Frequency Ratio (FR), being the ratio of the mean genotoxic parameters values of the exposed individuals to the corresponding control values, was calculated.

RESULTS

The studied group of pilots exposed to pesticides during aerial fumigations of agricultural fields in Sinaloa, Mexico was composed of 30 males aged 21 to 62 years, with a median of 38.0 years; and 30 male not involved (controls) in pesticide applications aged 21 to 59 years, with a median of 34.5 years. The comparison between medians ages revealed not statistical differences ($p > 0.05$). The results of Comet Assay comparisons are presented in table 2. The median of the comet's frequency, tail length (SE:159.6 $\pm$ 16.8) and tail moment (SE:16.75 $\pm$ 3.13), reveals statistically significant differences ($p < 0.001$) between the exposed pilots compared to the control group ($p < 0.001$). By computing the magnitudes of differences expressed by Frequency Ratios, highest values for tail moment (1.8) and tail length (1.5) were obtained.

Comet Assay is a valuable tool in measuring single and double-strand breaks of DNA and the extent of the damage caused by different types of genotoxic compounds. Cells with increased DNA damage display increased migration of chromosomal DNA from the nucleus toward the anode, which resembles the shape of a comet determining the level of DNA damage. This permits early evaluation of health risks in populations exposed to environmental toxic agents. The results of frequency of damage calculated for 50 comets according to Collins (2004) are presented in table 3 and figure 1.

To view whether the age of the exposed pilots can influence on the DNA damage intensity, the total group was divided in two subgroups according to age (21 years to 39 years and 40 years to 62 years). The obtained results are presented in table 4.

The obtained results of Anova, post hoc Tukey test and regression analyses expressed by R^2 values revealed age as an influencing factor. The results of DNA damage in human peripheral lymphocytes decreased when age increased. The values of frequency of comets, tail length and tail moment, were decreased statistically significant when age of participant increased. Our results are not in concordance with Araldi *et al.* (2015), Chen, Hales & Ozanne (2007), Heuser, Andrade, Peres, Gomes & Bogo (2008) who indicate that when age increases, DNA damage increases in parallel. Evaluating the FRs of tail length and tail moment, the genotoxic response decreased when age of participants increased. Some studies have shown an increase in the frequency of nuclear abnormalities with age (Bolognesi *et al.*, 1999; Fenech, 1998; Thomas, Harvey, Gruner & Fenech, 2008). According to Barnett & King (1995), the influence of age on genotoxic and cytotoxic endpoints may potentially reflect the increase in spontaneous chromosome instability with ageing, which in turn relates to an accumulation of DNA damage, due to a progressive impairment of the overall DNA repair capacity. Our results of statistical comparisons and a Frequency Ratio's analysis indicated that there is no significant association between the life years and DNA damage. In the cases of chronic exposure of the pilots studied, early DNA damage was noted to which the kinetics of cells replications did not affect the occurrence of DNA damages. Afterwards, chronic exposure leads to a steady state of an elevated expression of DNA and progressive DNA repair regardless of the cell division rate (Ceppi, Gallo & Bonassi, 2011), which was expressed by a decrease of tail length and tail moment values in elder pilots.

Table 2 Comparison of comet assay results and frequency ratios between exposed pilots and controls groups

Variable	Pilots exposed		Controls not exposed		Mann Whitney test	FR
	X $\pm$ SEM	Median	X $\pm$ SEM	Median		
Tail Length	159.6 $\pm$ 16.8	135.3	106.6 $\pm$ 7.7	98.1	0.001*	1.5
Tail Moment	16.75 $\pm$ 3.13	13.1	13.77 $\pm$ 1.91	10.41	0.839	1.2
Age	37.7 $\pm$ 3.1	38	35.9 $\pm$ 2.6	34.5	0.695	

* Statistically significant differences between medians.
Source: Author's own elaboration.

Table 3 Distribution of DNA damage frequencies observed in comet assay of pilots studied acording to 5 categories (0 - without damage, damage categories: 1, 2, 3 and 4)

Category of damage	0	1	2	3	4
Damage frequency	27.60%	27.20%	20.30%	10.20%	14.70%

Source: Author's own elaboration.

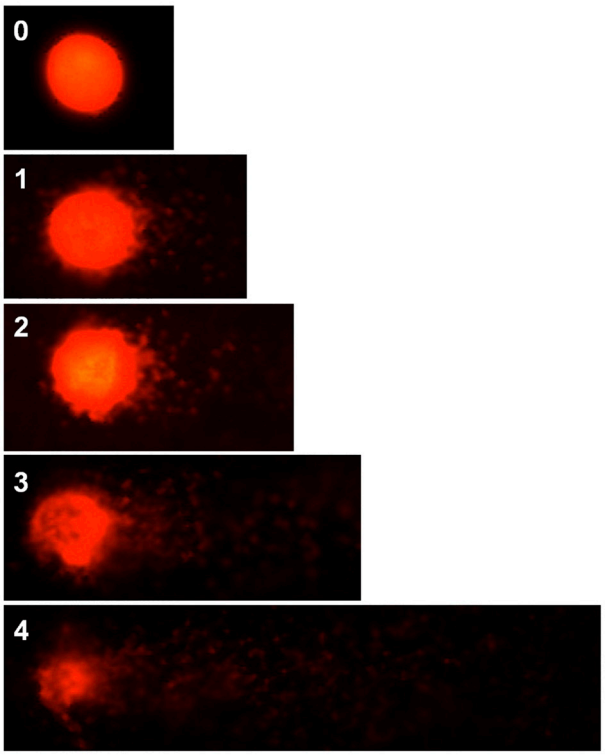

Figure 1

Human peripheral blood lymphocytes: 0: nuclei without DNA damage (without comet) and 1, 2, 3, 4: nuclei with ascending DNA damage. 400 X.
Source: Author's own elaboration.

Table 4 Comparison of comet assay parameters and frequency ratios between pilots divided into four subgroups according to the age

Variable	21 to 39 years	40 to 62 years	R^2	ANOVA	FR
	X ± SE	X ± SE			
Tail Length	192.9 ± 26.3	118.8 ± 7.6	25.36%	0.024*	0.6
Tail Moment	23.35 ± 4.75	8.68 ± 1.52	28.69%	0.015*	0.4
Age	27.3 ± 2.2	50.6 ± 2.2	75.42%	0.001*	
	Up to 4 years	5 to 8 years			
Tail Length	141.7 ± 11.0	177.4 ± 31.6	5.95%	0.05*	1.3
Tail Moment	13.35 ± 2.16	20.15 ± 5.84	6.22%	0.05*	1.5
Age	35.4 ± 4.2	40.1 ± 4.5	3.10%	0.46	

* $p < 0.05$ of Tukey test
Source: Author's own elaboration.

To see if years dedicated to pesticide applications have any relation to Comet Assay results, the exposed pilot group was divided into two subgroups according to years of work. The obtained results of the comparison are presented in table 4.

Anova comparison and post hoc Tukey test between exposed pilots up to 4 and from 5 to 8 years of work revealed a statistically significant increase of Comet Assay results, which indicate that there exists an influence of years of work and exposure period to DNA damage status. In other words, when the years of work increase, the time of exposition to aerial pesticide applications increase in parallel, enabling more DNA damage. The Frequency Ratios calculated for means of both groups showed an increase in magnitude of DNA damage.

To view if lifestyle habits such as tobacco consumption can potentiate the magnitude of DNA damage among the studied pilots, the total pilot population was divided according to the smoking habit: 19 no-smoking and 11 smoking pilots. The results of the comparisons are presented in table 5.

To observe possible differences between the studied groups, the means and medians were compared indicating a significant higher Comet Assay expression ($p < 0.05$) of non-smoking participants and higher genotoxic results (table 6).

Finally, to compare if alcohol consumption can have an influence on Comet Assay results, the pilot group was divided into drinkers ($n = 13$) and no-drinkers ($n = 17$). The results of these comparisons are presented in table 7.

Table 5 Comparison of comet assay results and frequency ratios between pilots divided in four groups according to the tobacco smoking habit and alcohol consumption habit

Variable	No-smoking $n = 19$		Smoking $n = 11$		FR
	X ± SE	Median	X ± SE	Median	
Tail Length	167.0 ± 21.3	135.7	137.2 ± 20.9	120	1
Tail Moment	18.10 ± 3.94	13.15	12.70 ± 4.20	8.47	1
Age	39.9 ± 3.4	43	31.2 ± 6.5	26	
Years of Work	5.2 ± 0.3	5	4.3 ± 0.3	4	

Source: Author's own elaboration.

Table 6 Comparison of comet assay results and frequency ratios between pilots divided into two groups according to tobacco smoking habit

Variable	No-smoking $n = 19$		Smoking $n = 11$		FR
	X ± SE	Median	X ± SE	Median	
Tail Length	167.0 ± 21.3	135.7	137.2 ± 20.9	120	1.2
Tail Moment	18.10 ± 3.94	13.15	12.70 ± 4.20	8.47	1.4
Age	39.9 ± 3.4	43	31.2 ± 6.5	26	
Years of Work	5.2 ± 0.3	5	4.3 ± 0.3	4	

Source: Author's own elaboration.

Table 7 Comparison of comet assay results and frequency ratios between pilots divided into two groups according to the alcohol consumption habit

Variable	No-drinkers $n = 17$		Drinkers $n = 13$		FR
	X ± SE	Median	X ± SE	Median	
Tail Length	166.7 ± 24.8	134.9	146.2 ± 14.9	156.1	1.1
Tail Moment	18.19 ± 4.61	9.4	14.08 ± 2.76	13.15	1.3
Age	38.5 ± 4.6	45	36.4 ± 2.4	37	
Years of Work	5.2 ± 0.4	5	4.3 ± 0.4	4	

Source: Author's own elaboration.

The mean and median comparison tests and Frequency Ratios revealed significant differences between no-drinkers and drinkers ($p < 0.05$) with higher values of no-drinkers and no influence of alcohol consumption on genotoxic parameters of Comet Assay. The no-drinkers corresponds to the older pilots (median 45.0 years old) and higher years of work (median 5.0 years old), factors which justified a more prolonged exposition period which caused higher genotoxic damages expressed in Comet Assay results. In general, the results of our study showed alcohol consumption as a non-influencing factor on DNA damage.

DISCUSSION

Occupational pesticide poisoning represents a major potential health hazard for sprayers in agricultural fields. Prolonged exposure to pesticides has been linked to increased risk of chronic diseases such as Parkinson's (Moisan *et al.*, 2015; Tingting *et al.*, 2017), Alzheimer's (Cabrera, 2017; Richardson *et al.*, 2014) multiple sclerosis (Parrón, Requena, Hernández & Alarcón, 2011), diabetes, and cardiovascular and renal diseases (Gangemi *et al.*, 2016). Chronic pesticide exposure due to the use of large quantities of agrochemicals at the fields are the most prevalent and serious occupational hazards of workers in many countries (Falzone *et al.*, 2016). A drift of applied pesticides, originated during fumigations, can cause severe health effects in the involved persons.

The oxidative stress induced by pesticide exposures as a possible mechanism of toxicity and human health uncertainty has been the focus of toxicological researchers for over a decade. Given that DNA damage is one of the primary mechanisms for the progress of some of these chronic diseases, genotoxicity biomarkers have been suggested as predictors of risk for the expansion of cancer (Collins *et al.*, 2014). Genetic biomonitoring is also important since it enables the identification of risk factors at a time when control measures may still be implemented. Studies on DNA damage in workers exposed to pesticides present both positive as well as negative results which may reflect different exposure conditions such as the intensity of exposure, the use of personal protective equipment, the specific genotoxic potential of the pesticides used and worker health status. A primary risk factor of genotoxic agrochemicals is that they act directly or indirectly causing chronic genotoxicity (Guanggang *et al.*, 2013).

The Comet Assay analyze the genotoxicity in specific samples, which are in direct contact with the tested chemicals or in which the absorption, distribution, metabolization or excretion occur, allowing to detect the clastogenicity in situ. Comet Assay also allows detecting breaks in DNA strands, which can be visualized by the increased migration of free DNA segments, resulting in images similar to comets (Azqueta & Collins, 2013). These breaks are associated to chromosomal aberrations and genomic instability (Pfeiffer, Goedecke & Obe, 2000), directly associated to malignancy (Araldi *et al.*, 2015; Charames & Bapat, 2003; Eyfjord & Bodvarsdottir, 2005; Hanahan & Weinberg, 2011; Negrini, Gorgoulis & Halazonetis, 2010).

The results of Yaduvanshi *et al.* (2012) caused a highest increase in the DNA damage compared to individual pesticides treatments. The observed synergistic effect might be due to the combined impact of these complementary pesticides simultaneously acting in different ways and, thus, magnifying their toxicity. The genotoxic potential of pesticides mixture used habitually in agricultural fields was found to be synergistic and, therefore, may pose greater mutagenic risk to exposed individuals such as pilots applying different pesticides during one working day

and being exposed to the pesticide mixture. Certain pesticides are capable to interact chemically when they are combined in mixtures, mainly because the metabolism of one chemical can affect the metabolism of the other. Thus, it is possible that mixtures of pesticides can produce additive or synergistic effects. Combined exposure of non-toxic doses can induce significant frequencies of chromatid breaks and fragments, whereas independent exposure of similar doses failed to induce any effect. These studies indicate that pesticides used, which can form mixtures during aerial fumigations on Sinaloa agricultural fields, show an increase in the frequencies of chromosomal aberrations, DNA adducts formation that can disrupt the genetic integrity and alter the metabolism and other vital functions of the cell or organism (Yaduvanshi *et al*., 2012), perhaps associated with generation of oxidant stress. Diverse studies have shown that organophosphorus, carbamate, organochlorines, pirethroids pesticides stimulate ROS production or generate more toxic compounds. ROS, such as superoxide anions ($O_2^{\bullet}$), hydrogen peroxide (H_2O_2), and hydroxyl radicals (OH^-), are highly reactive with DNA and produce diverse kind of damage, including single and double-strand DNA breaks, nucleoside modifications, mutations and apoptosis which are associated in the process of carcinogenicity (Valko, Rhodes, Moncol, Izakovic & Mazur, 2006). Possibly, the results expressed by higher DNA damage in human peripheral lymphocytes confirmed the cytotoxic effect of pesticides by assaying for apoptosis-associated fragmentation of nuclear DNA (Jha, 2008; Li *et al*., 2015).

In the present study we used a software program to objectively describe and measure comet sizes and, thereby, to eliminate the need for subjective visual estimation (e.g., the presence or absence of a comet shape). In general, for both Comet Assays and dimension measures (comet and tail length, comet height, head diameter and area) had the least variability, while intensity measures were the greatest. Comet Assay dimension measures used in our study are more repeatable than intensity and moment (i.e., tail/Olive) measures. The moment measures are more likely to exhibit variability because they incorporate intensity, a highly variable measure, into the value (Serafini, Romano, Varner, Di Palo & Love, 2015).

In this study, the visual appearance and the length of the comet tails showed marked differences between exposed pilots and controls, reflecting DNA breaks (i.e., higher tail length – 1.5) in samples from exposed pilots compared to those of controls, results that can be observed in the tables of the results section. The comets were rounded shape which suggested that DNA remains in a more compact form, resulting in fewer actual comet shapes. The head diameter and area were smaller and less intense; suggesting that less DNA remained in the nucleoid. Therefore, DNA that is more fragmented prior to analytical process may result in the production of smaller fragments that may disappear visually (Simon & Carrell, 2013).

Measurements made by image analysis and DNA migration are expressed by the tail intensity (% tail DNA). This approach might be particularly useful in human biomonitoring, because relatively low DNA damage levels can usually be expected after occupational and environmental exposure to genotoxic agents (Bausinger & Speit, 2014).

The study found that the incidence of drift-related pesticide poisoning has influenced on pilots who do aerial spraying of pesticide on Sinaloa agricultural fields. These groups of agricultural workers were found, by Calvert *et al.* (2008), to have a higher incidence of poisoning pesticide. It is not known why the incidence occurs but the Comet Assay results show higher DNA damage among pilots studied, indicating that they are at greater risk of exposure, and that they can be more susceptible to pesticide toxicity, or that they can more likely report exposure and illness or seek medical attention (Lee *et al*., 2011).

These findings, which are limited only to 30 exposed pilots and 30 controls, presented higher incidence of Comet Assay results from monitored pilots and DNA damage. The rate of poisoning from inhaled drift of applied pesticides was many times higher for occupationally exposed applicators in agricultural fields than in nonagricultural workers that constituted the controls.

The genotoxicity studies of chemical compounds require special attention to the age of the biological material donor. Extensive observations suggest that DNA damage accumulates with age (Chen *et al*., 2007; Heuser *et al*., 2008). These data are not similar to those obtained in this study, where age of participants was not calculated as an influential factor on Comet Assay results and degree of DNA damage intensity, suggesting only worked and exposure period as significant factors in evaluation of DNA damage degree.

Comet Assay can be used to assess apoptosis in a general term and to describe a non-inflammatory programmed cell death in contrast with frequently non-programmed and highly inflammatory necrosis. Several features confer to apoptotic cells a peculiar signature allowing them to be discriminated from cells compromised with other non-apoptotic mechanisms (Kepp, Galluzzi, Lipinski, Yuan, & Kroemer, 2011). Meanwhile, currently several methods aimed at identifying DNA damages may be performed, and Comet Assay stands out as a potential alternative to evaluate DNA injuries, mainly due to easy implementation and low costs. The results provide valuable information at the stage of potentially degenerative diseases on the early

pre-symptomatic diagnosis. A combination of nuclear alterations with the development of various diseases suggests that the used assay could potentially play an important role in identifying individuals, such as pilots applying pesticides as a group, with a higher risk to develop diseases originated from nuclear instability. These results make clear that pesticide exposition increases frequencies of cells with nuclear damage significantly, due to the genotoxic effect of inhaled pesticide vapors to which they are exposed.

CONCLUSION

The present results indicate a significant association between the occupational exposure to managed pesticides and the occurrence of nuclear damages determined by Comet Assay. The use of biomarker studies reflects the potential alterations in DNA kinetics and metabolism viewed in the genomic instability events. The results provide valuable information at the stage of potentially degenerative diseases on the early pre-symptomatic diagnosis. A combination of Comet Assay results with the development of various diseases suggests that the used assay could potentially play an important role in identifying individuals, such as pilots applying pesticides, who have a higher risk todevelop diseases originated from nuclear instability. These results make clear that pesticide exposition increase significantly frequencies of nuclear abnormalities, due to the genotoxic effect of inhaled pesticide vapors to which they are exposed.

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